09/868744

PCT/GB 99/04317

16 DECEMBER 1999

The Patent Office

INVESTOR IN PEOPLE

GB99/4317

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

REC'D 26 JAN 2000
WIPO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

09/868744.

Signed

Dated 12 January 2000

ents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
D
21 DEC 1998
RECEIVED

The Patent Office

1/77

22DEC98 E413603-1 D01874
P01/7700 0.00 - 9828201.5

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference — TR/RM/98102GB

2. Patent application number *(The Patent Office will fill in this part)* — 21 DEC 1998 — **9828201.5**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Zenco (No. 4) Limited
15 Stanhope Gate
London
W1Y 5TG

Patents ADP number *(if you know it)* 7211035001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

GENETIC MODIFICATION OF COMPOSITAE

5. Name of your agent *(if you have one)*

Batchellor, Kirk & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

2 Pear Tree Court,
Farringdon Road,
London EC1R ODS.

Global change
102 Clerkenwell Road
LONDON
EC1M 5SA

Patents ADP number *(if you know it)* 315001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	0
Description	10
Claim(s)	0
Abstract	
Drawing(s)	3 & 3

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Batchellor, Kirk & Co. Date 21.12.1998

12. Name and daytime telephone number of person to contact in the United Kingdom

Mr. D.S. Weitzel

0171 253 1563

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

TITLE: GENETIC MODIFICATION OF *COMPOSITAE*

The present invention relates to the genetic modification of plants of the family *Compositae*, in particular lettuce (*Lactuca sativa*) and sunflower (*Helianthus annuus*).

Genetic modification of plants is now well established as an experimental technique, and increasingly such plants are being used in agriculture in different parts of the world. The technique offers a number of advantages. The introduction of heterologous genes allows the plant to produce heterologous proteins with functions that the plant does not normally possess. For example, introduction of the gene for production of the *Bacillus thuringiensis* (Bt) insecticidal protein renders the plant toxic to insects and protects it against insect attack. Similarly genes may be introduced which protect against the attack of fungal and viral diseases. It is also possible by genetic modification to control the expression of homologous genes which the plant already possesses. Thus by inserting extra copies of homologous genes under the control of a suitable promoter, the expression of the protein produced by such genes can be up-regulated. Correspondingly, down-regulation may be induced by means such as anti-sense technology, in which an inverted copy of the homologous gene is inserted in the plant. Expression of the inverted gene produces antisense RNA, which inhibits the expression of the natural gene. In this way, for example, ripening of fruit such as tomatoes has been delayed by inhibiting the action of the polygalacturonase gene.

The genetic modification of plants in this way offers many potential benefits to farmers, consumers and the environment. To farmers it offers the opportunity to avoid the labour and expense of chemical sprays; for consumers it can provide cheaper food of higher quality; it can protect the environment both by raising productivity (thereby

reducing pressure on agricultural land) and by reducing the amount of chemical pesticide introduced into the ecosphere.

A number of methods for transforming crop plants, both monocotyledons and dicotyledons, are now well-known. They include, for example, the ballistic method (the 'gene gun') in which heavy metal pellets, for example of gold or tungsten, are coated with DNA and fired into plant cells: and the Ti plasmid method. Another important requirement for producing useful transformed plants is the availability of an effective plant gene promoter. Many plant gene promoters are known: one very frequently used constitutive promoter is the CaMV (cauliflower mosaic virus) 35-S promoter. However not all such promoters are found to be equally effective in all plants. In some plants, in particular *Compositae*, for example lettuce (*Lactuca sativa*) and sunflower (*Helianthus annuus*), many heterologous constructs are found to have unstable expression levels. Both in primary transformants and in progeny, 'gene silencing' often causes a severe reduction in recombinant gene activity. In consequence, the plant reverts to a wild-type phenotype. For practical applications of gene technology this is unacceptable, and presents a real obstacle to the use of recombinant gene technology with plants such as lettuce and sunflower.

According to the present invention, therefore, we provide a method of producing a genetically-modified *Compositae* plant which comprises transforming the plant with a heterologous DNA construct including a DNA sequence adapted to express RNA in the plant under the control of the actin2 (ACT2) gene promoter. We further provide genetically-modified *Compositae* plant cells comprising a heterologous DNA construct including a DNA sequence adapted to express RNA in the plant under the control of the actin2 (ACT2) gene promoter; and *Compositae*, in particular lettuce and sunflower, plants comprising such cells.

The promoter of the actin2 (ACT2) gene derived from *Arabidopsis* has been shown to drive the beta-glucuronidase (GUS) gene in transgenic *Arabidopsis* plants to relatively high levels in vegetative tissues. In the inflorescence a developmental factor seemed to be involved causing a more differentiated expression pattern This is reported by An, Meagher *et al.* (1996), The Plant Journal 10, 107-121: the same reference gives the DNA sequence of the actin-2 promoter (p.109).

The invention will be further described with reference to the drawings, in which:

Figure 1 is a map of a construct pVDH380 comprising the promoter of the actin2 (ACT2) gene derived from *Arabidopsis* arranged to drive the beta-glucuronidase (GUS) gene.

Figure 2 is a map of a construct pVDH641comprising the promoter of the actin2 (ACT2) gene derived from *Arabidopsis* arranged to drive the OXOX gene of wheat;

Figure 3 gives the DNA sequence of the actin2 (ACT2) promoter derived from *Arabidopsis*.

Actin is a fundamental cytoskeletal component essential to nearly all eukaryotic cells, in which it forms microfilament structures. There are large families of plant actin genes, with greater diversity than corresponding animal genes. The actin 2 gene promoter is a constitutive promoter to be found in most plants. We particularly prefer to use the actin 2 gene promoter obtained from *Arabidopsis thaliana*: though corresponding actin 2 promoters can readily be isolated from other sources, particularly other plants, and used for the same purpose.

The DNA sequence which expresses RNA may be of two main kinds: either a sequence which expresses mRNA which is

translated into protein, or a sequence which produces RNA which is not translated into protein, but which interacts with the biochemistry of the plant cell in another way, for example by inhibiting gene expression.

A preferred use of the present invention is to promote the expression of heterologous genes. However it may also be used to up-regulate or down-regulate the expression of homologous genes. As heterologous genes may be used DNA sequences coding for insecticidal proteins (for example the Bt protein) or fungicidal or antiviral proteins. ACT2 can be used to drive oxox (the oxalate oxidase gene), giving sclerotinia resistance, or other genes like early or late flowering genes (for example ATH1), herbicide resistance genes, insect tolerance genes (aphid resistance in lettuce), virus resistance genes (eg lettuce mosaic virus, LMV), nitrate reductase to lower nitrate content, and genes for increased shelf life. Many traits desirable in lettuce and sunflower can be exploited using this promoter.

The use of DNA sequences of homologous genes to inhibit or promote gene expression is quite well understood. A complete gene sequence, under the control of a suitable promoter, that operates effectively in the plant, will generally overexpress the gene product, leading to an amplification of the effect of the protein so produced. Sometimes the gene product is reduced: this phenomenon is termed "co-suppression". Reduction of the gene product is also generally obtained by reversing the orientation of the gene sequence with respect to the promoter so that it produces "antisense" messenger RNA.

A DNA construct for use in the invention may be an "antisense" construct generating "antisense" RNA or a "sense" construct (encoding at least part of the functional protein) generating "sense" RNA. "Antisense RNA" is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base

(or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. Such antisense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged to generate a transcript with at least part of its sequence complementary to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). "Sense RNA" is an RNA sequence which is substantially homologous to at least part of the corresponding mRNA sequence. Such sense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged in the normal orientation so as to generate a transcript with a sequence identical to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). Suitable sense constructs may be used to inhibit gene expression (as described in International Patent Publication WO91/08299) or a sense construct encoding and expressing a homologous functional protein may be transformed into the plant to over-express the protein.

DNA constructs for use in the invention to inhibit gene expression may comprise a base sequence at least 10 bases (preferably at least 35 bases) in length for transcription into RNA. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length.

As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA or synthetic polynucleotide may be used.

The invention will be further described with reference to the following Examples.

EXAMPLES

Lettuce was transformed with various constructs comprising the act2 gene promoter. The act2/GUS construct shown in Figure 1, kindly provided by courtesy of Dr. R. B. Meagher, Dept. of Genetics, University of Georgia, Athens, GA 30602, USA, and by us termed pVDH380 (Figure 1), was used to transform lettuce, in order to evaluate the expression pattern in primary transformants as well as the stability in consecutive generations. The construct pVDH380 was used to make a construct pVDH641 (Figure 2) containing the act2 promoter linked to the oxalate oxidase (OXOX) gene of wheat: this construct was used in transient expression studies in both lettuce and sunflower.

- *Results*

ACT2/GUS in lettuce

The binary vector pVDH380 of Figure 1 contains the NPTII gene as a selectable marker in addition to the ACT2/GUS gene. The act2 promoter sequence includes the first 19 codons of the act2 gene as well as the first exon-intron combination. Vector pVDH380 was used directly to transform lettuce (variety "Evola"), following the Ti plasmid method given in Curtis *et al.* (1994), J. Exp. Bot. **45**, 1441-1449.

From this transformation experiment were obtained a total of 38 independent transformants displaying a wide range of GUS-activities (as judged from a histochemical GUS staining using leaf explants of greenhouse grown material). These were compared with control CaMV 35S-GUS transformants, prepared similarly. The act2/GUS transformants of the invention showed higher and more uniform levels of GUS activity than the CaMV controls.

Subsequently, twelve independent act2/GUS transformants were used to carry out further histochemical assays. Tissues were taken from leaves, stems, roots, and flowers (sepals, petals, stamens, carpels). The tissues examined showed

consistent GUS activity levels for nine of the events. Three of the events however showed a certain degree of variability in expression (e.g. enhanced in flowers or vegetative tissues) which is probably due to the site of integration in the genome.

It was concluded that the act2/GUS construct displays a relatively strong, predominantly constitutive expression pattern in transgenic lettuce at the T0 level.

Seeds were harvested and a total number of 15 events were analyzed in the T1 generation using greenhouse grown material. The segregation data in the T1 generation, as well as levels of GUS activity in the T0 generation, are shown in Table 1 below.

TABLE 1

Segregation analysis of transgenic lettuce expressing ACT2/GUS		
Transformant code	*GUS activity in T0*	*Segregation of GUS activity in T1*
Ev-1A-1	+	66+ / 17-
Ev-1A-3	++	20+ / 7-
Ev-2A-2	+	82+ / 24-
Ev-2A-4	-	0+ / 97-
Ev-4A-3	++	75+ / 22-
Ev-5A-2	-	0+ / 102-
Ev-5A-4	-	0+ / 97-
Ev-7A-1	-	0+/ 92-
Ev-8A-1	++	87+ / 22-
Ev-10A-1	+	75+ / 19-
Ev-10A-3	+	58+ / 20-
Ev-12A-1	+	36+ / 13-
Ev-13A-2	++	84+ / 3-
Ev-14A-1	++	78+/ 17-
Ev-14A-2	-	0+ / 98-

"+" = active; "++" = highly active; - = "not detected"

The most important observation is that there is no significant loss of GUS activity when the gene is transferred to the T1. This contrasts sharply with the situation in which 35S-GUS is used which typically results in a total inhibition of gene activity in 90% of the events during transmission from one generation to the next.

EXAMPLE 2

act2/OXOX in lettuce and sunflower

A further experiment was done to illustrate the use of the act2 promoter to drive other genes besides GUS. As the act2/GUS construct pVDH380 (Figure 1) contains 19 codons of the actin2 gene it was decided to modify the promoter by PCR

using a primer combination which generates a unique restriction site at the act2 transcription start. This modified promoter was fused to the OXOX gene and inserted into a binary vector, as described below.

. Construction of ACT2-OXOX

Starting material for the ACT2-OXOX construct of Figure 2, termed pVDH641 (Figure 2), was the plasmid pVDH380 (Figure 1) which is identical with the plasmid ACT2/GUS described by An et al., cited above.

Figure 1 shows a physical map of the construct pVDH380. In this figure, 'LB' indicates the left border, 'RB' indicates the right border, 'Pnos' indicates the nopaline synthase promoter, 'Tnos' indicates the nopaline synthase terminator, 'NPTII' indicates the neomycin phosphotransferase II gene, 'pACT2' indicates the Actin 2 promoter, 'GUS' indicates the beta-glucuronidase gene and 'KanR' indicates the bacterial kanamycin resistance gene.
The ACT2 promoter was recloned from vector pVDH380 after amplification by PCR.

Figure 3 shows the nucleotide sequence of the Actin 2 promoter region. The sequence corresponding to the forward primer (bold, Gothic typeface) as well as to the complementary sequence (bold underlined) of the backward primer are indicated. The start codon of the Actin 2 gene, ATG is given in bold capitals. In addition, the composition of the forward and backward primers are given. We used a primerset consisting of primer 1 (5'-GC AAGCTT ATT ATG ATC TCA AAT ACA TTG-3') and primer 2 (5'-GC GGATCC TTT ATG AGC TGC AAA CAC AC-3'). Primer 1 contains after the first two nucleotides a HindIII restriction recognition site and subsequently a nucleotide sequence identical to the nucleotide sequence located from position 1358 to position 1379 upstream from the ATG-start codon (see Figure 3). Primer 2 contains after the first two nucleotides a BamHI restriction recognition site and subsequently 20 nucleotides complementary from position 3 to position 22 upstream from the start codon. The

DNA fragment which was obtained after amplification was digested with HindIII and BamHI and inserted in the vector pVDH478. pVDH478 is a binary vector containing between the left and right border the NPTII gene, flanked upstream by the nopaline synthase promoter and downstream by the nopaline synthase poly(A)-signal. It also contains the coding region of the oxalate oxidase gene (OxOx) with its own poly(A)-signal which is derived from wheat(for more information on the OxOx gene, including sequence data, see PCT Publication WO92/14824). The resulting vector was called pVDH641. A physical map of pVDH641 is shown in Figure 2. in this Figure, annotations in common with Figure 1 have the same meaning as in that Figure. Additionally, 'TOxOx' indicates the oxalate oxidase terminator, and 'OxOx' indicates the oxalate oxidase gene. The main restriction enzymes are indicated.

Sequence analysis confirmed that the ACT2 promoter region had been inserted without any mutation having occurred during the PCR amplification. OxOx activity can be measured in a histochemical assay using oxalate which is converted by the enzyme into a purple dye. The act2/OXOX
fusion showed good levels of OxOx activity in transient assays, using both lettuce and sunflower explants, confirming the functionality of the construct.

twr 15/12/98

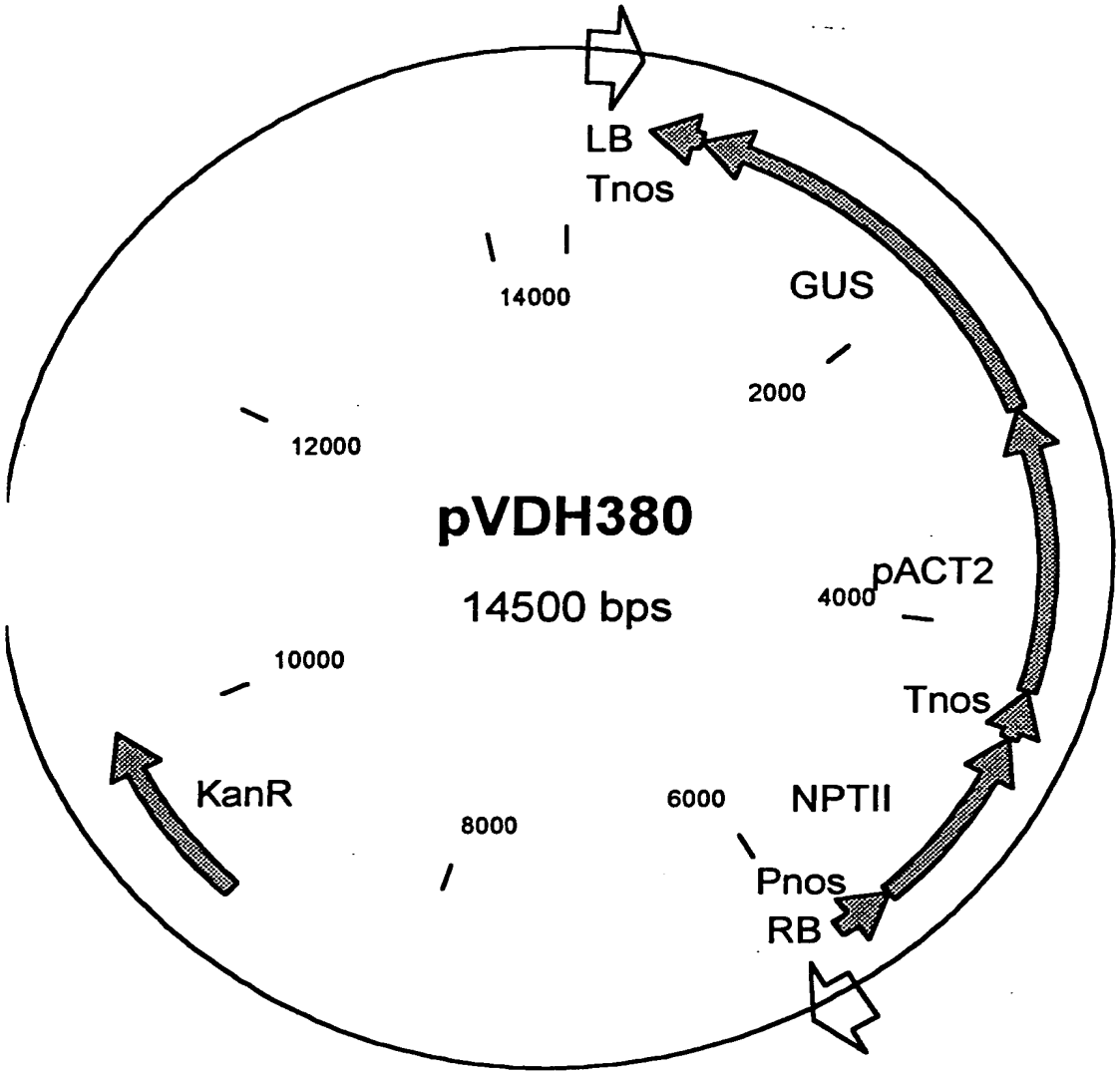
LB
Tnos
14000
GUS
2000
12000
pVDH380
14500 bps
pACT2
4000
10000
Tnos
KanR
6000
NPTII
8000
Pnos
RB

Figure 1

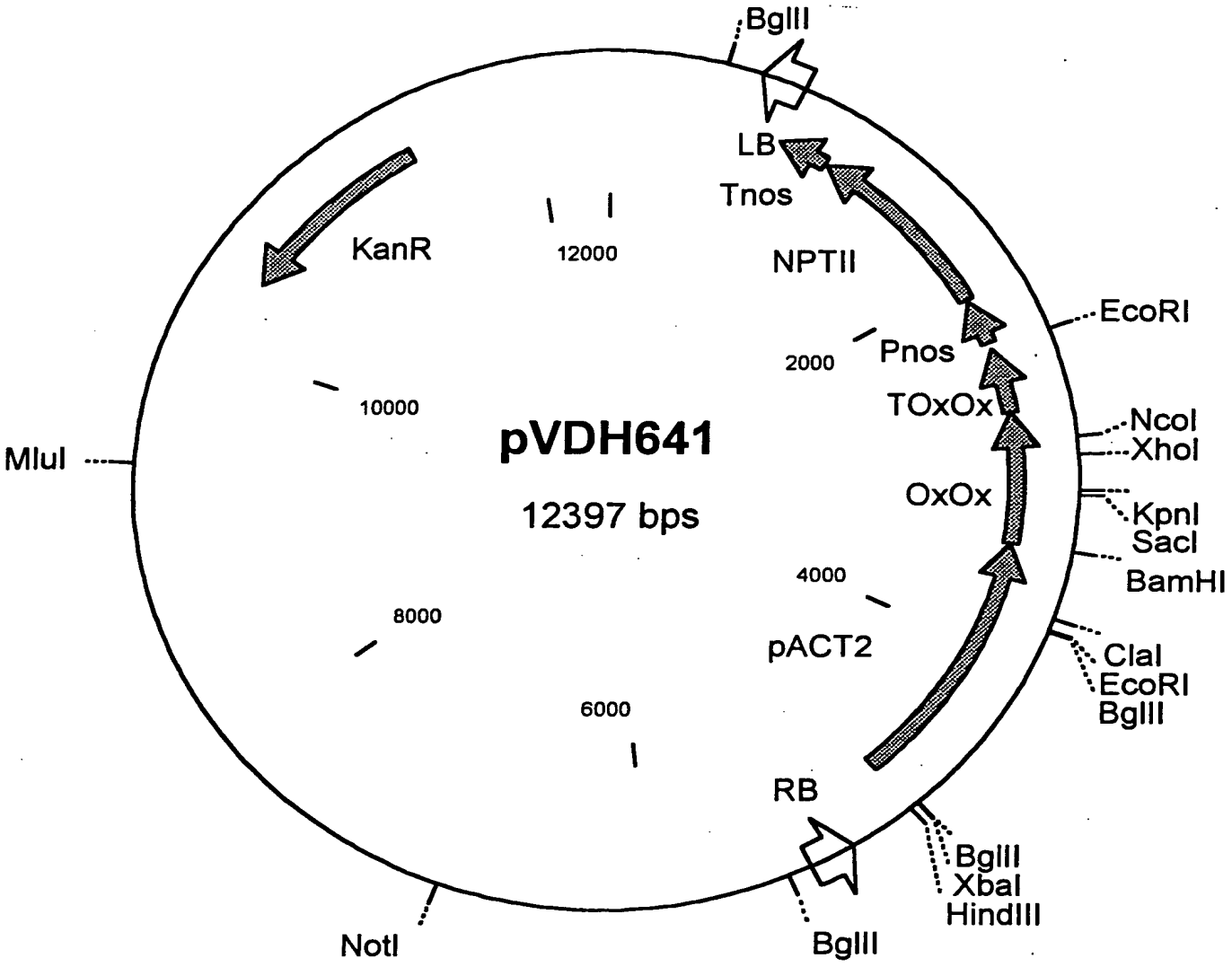
BglII
LB
Tnos
NPTII
KanR
12000
2000
Pnos
EcoRI
10000
TOxOx
NcoI
XhoI
MluI
pVDH641
12397 bps
OxOx
KpnI
SacI
BamHI
4000
8000
pACT2
ClaI
EcoRI
BglII
6000
RB
BglII
XbaI
HindIII
NotI
BglII

Figure 2

```
   1  attatgatct caaatacatt gatacatatc tcatctatc
  41  ctaggttatc attatgtaag aaagttttga cgaatatgnn
  81  acgacaaaat ggctacactc gatgtaattg gtatctcaac
 121  tcaacattat acttatacca aacattagtt agcaaaattt
 161  aaacaactat ttttatgtat gcaagagtca gcatatgtat
 201  aattgattca gaatcgtttt gacgagttcg gatgtagtag
 241  tagccattat ttaatgtaca tactaatcgt gaatagtgat
 281  atgatgaaac attgtatctt attgtataaa tatccataaa
 321  cacatcatga aagacacttt ctttcagggt ctgaattaat
 361  tatgatacaa ttctaataga aaacgaatta aattacgttg
 401  aattgtatga aatctaattg aacaagccaa ccacgacgag
 441  gactaacgtt gcctggattg actcggttta agttaaccac
 481  taaaaaaacg gagctgtcat gtaacacgcg gatcgagcag
 521  gtcacagtca tgaagccatc aaagcaaaag aactaatcca
 561  aggggtgaga tgattaatta gtttaaaaat tagttaacac
 601  gagggaaaag ctgtctgaca gccaggtcac gttatcttta
 641  cctgtggtcg aaatgattcg tgtctgtcga ttttaattat
 681  ttttttgaaa ggccgaaaat aaagttgtaa gagataaacc
 721  cgcctatata aattcatata ttttcctccc cgctttgaat
 761  tgtctcgttg tcctcctcac tttcatcagc cgttttgaat
 801  ctccggcgac ttgacagaga agaacaagga agaagactaa
 841  gagagaaagt aagagataat ccaggagatt cattctccgt
 881  tttgaatctt cctcaatctc atcttcttct tccgctcttt
 921  ctttccaagg taataggaac tttctggatc tactttattt
 961  gctggatctc gatcttgttt tctcaatttc cttgagatct
1001  ggaattcgtt taatttggat ctgtgaacct ccactaaatc
1041  ttttggtttt actagaatcg atctaagttg accgatcagt
1081  tagctcgatt atagctacca gaatttggct tgaccttgat
1121  ggagagatcc atgttcatgt tacctgggaa atgatttgta
1161  tatgtgaatt gaaatctgaa ctgttgaagt tagattgaat
1201  ctgaacactg tcaatgttag attgaatctg aacactgttt
1241  aagttagatg aagtttgtgt atagattctt cgaaaccttа
1281  ggatttgtag tgtcgtacgt tgaacagaaa gctatttctg
1321  attcaatcag ggtttatttg actgtattga actcttttтg
1361  tgtgtttgca gctcataaaa aATGgctgag gctgacgata
1401  ttcaaccaat cgtgtgtgac aatggtactg gaatggtagg
1441  atcc
```

HindIII

Actin 2 primer forward. GC AAGCTT attatgatct caaatacatt g

BamHI

Actin 2 primer backward GC GGATCC tttatgagctgcaaacacac

Figure 3

DCT NO: 99/04317

Form 23/77 : 16/12/99

Agnt : Batchellor Kirk & Co.